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DOI:

[10.1016/j.archoralbio.2016.05.013](https://doi.org/10.1016/j.archoralbio.2016.05.013)

Document Version

Peer reviewed version

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Citation for published version (APA):

Teshima, T. H.N., Ianez, R. C.F., Coutinho-Camillo, C. M., Tucker, A. S., & Lourenço, S. V. (2016). Apoptosis-associated protein expression in human salivary gland morphogenesis. *Archives of Oral Biology*. <https://doi.org/10.1016/j.archoralbio.2016.05.013>

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Accepted Manuscript

Title: Apoptosis-associated protein expression in human salivary gland morphogenesis

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PII: S0003-9969(16)30123-6
DOI: <http://dx.doi.org/doi:10.1016/j.archoralbio.2016.05.013>
Reference: AOB 3606

To appear in: *Archives of Oral Biology*

Received date: 9-9-2015
Revised date: 13-5-2016
Accepted date: 15-5-2016

Please cite this article as: Teshima Tathiane HN, Ianez Renata CF, Coutinho-Camillo Claudia M, Tucker Abigail S, Lourenço Silvia V. Apoptosis-associated protein expression in human salivary gland morphogenesis. *Archives of Oral Biology* <http://dx.doi.org/10.1016/j.archoralbio.2016.05.013>

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Apoptosis-associated protein expression in human salivary gland morphogenesis

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Running title: Apoptosis in foetal salivary gland

Keywords: salivary glands, human, development, apoptosis, caspases, bcl-2 family

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Abstract

Objective: Salivary gland (SG) development is based on branching morphogenesis, in which programmed cell death has been proposed to play a role in cell signalling and organ shaping. In the mouse salivary gland apoptosis has been suggested to play a key role in lumen formation, removing the central cells of the epithelial stalks. Here we analyse the expression of several anti- and pro-regulators of apoptosis during human SG development in a range of developmental stages.

Design: Foetal SGs obtained from the University of São Paulo were analysed by immunohistochemistry to assess the expression of apoptosis-associated proteins: caspases (caspase-6, -7, -9 and cleaved caspase-3), Bcl-2 family members (Bax, Bak, Bad, Bid, Bcl-2, Bcl-x and Bcl-xL), Survivin (BIRC5), Cytochrome C and Apaf-1.

Results: Nuclear expression of Bax and Bak was identified in presumptive luminal areas at initial stages, while Bcl-xL showed the most relevant anti-apoptotic activity. Caspase-6, -7 and -9 were expressed during all stages, while interestingly cleaved caspase-3 showed no prominent expression, indicating that caspase-7 is the main effector. Apoptosome complex components Apaf-1 and Cytochrome C, as well as survivin were all positive in developing glands.

Conclusions: The particular expression pattern of several apoptotic regulators in human SG development suggests the existence of a fundamental role for apoptosis during duct formation. The absence of Bad and Bid expressions indicates that the intrinsic pathway is more active than the extrinsic during human gland formation. The subcellular localisation of intrinsic-apoptosis proteins correlated with apoptotic activity, but also suggested additional non-apoptotic functions.

Highlights:

- Caspase-7 is the main executioner caspase involved in human SG duct development
- Consistent cytoplasmic expression suggest additional non-apoptotic functions
- Intrinsic-type apoptotic pathway drives human SG development

Introduction

The development of functionally complex and well-ordered salivary glands (SG) requires epithelial-mesenchymal interaction that depends on a variety of cellular signalling, growth factors and associated extracellular matrix components (Harunaga, Hsu, & Yamada, 2011; Patel, Rebustini, & Hoffman, 2006). SG development has classically been divided into five embryonic stages, well described as prebud, initial bud, pseudoglandular, canalicular (or cavitation), and terminal bud, observed in both mouse and human development (Melnick & Jaskoll, 2000; Teshima et al., 2011; Tucker, 2007). Apoptosis is a type of programmed cell death, which has been shown to be very important for the regulation of developmental and pathological processes (Elmore, 2007). Its role in gland morphogenesis is associated with the elimination of the central cells within solid epithelial stalks in order to create lumens (Andrew & Ewald, 2010; Debnath et al., 2002; Mailleux et al., 2007; Melnick & Jaskoll, 2000; Wells & Patel, 2010). In keeping with this, inhibition of apoptosis during mouse SG development *in vitro* has recently been shown to impair lumen formation (Teshima et al., 2016). Despite this knowledge from studies in the mouse, a comprehensive analysis of the expression of apoptosis regulators in human glands has not been previously carried out.

Different molecular pathways can trigger the complex apoptosis cascade (Suzanne & Steller, 2013). The extrinsic pathway (death-receptor pathway) involves transmembrane interactions mediated by apoptotic signalling from neighbouring cells that signal through receptors from the tumour necrosis factor superfamily (TNFr)(3). The intrinsic pathway in contrast does not depend on extracellular regulators, although sometimes a crosstalk can occur between them by Bid and caspase-8 (Korsmeyer et al., 2000; H. Li, Zhu, Xu, & Yuan, 1998). Diverse intracellular stress such as DNA damage, hypoxia, oncogene activation and deprivation of growth factors can activate this pathway, and mitochondria is the central organelle involved, acting through the release of several pro-apoptotic molecules into the cytoplasm (Danial & Korsmeyer, 2004).

The intrinsic apoptotic pathway is mainly triggered by Bcl-2 (B cell lymphoma 2) family proteins; the balance between pro and anti-regulators defines cell fate (Chipuk, Moldoveanu, Llambi, Parsons, & Green, 2010; Elmore, 2007; Martinou & Youle, 2011). Bax and Bak proteins are the main pro-apoptotic members that essentially modify the mitochondrial membrane permeability. Upon activation by endogenous death signals, Bax and Bak undergo oligomerization and promote the release of contents of the mitochondrial intermembrane space such as cytochrome c, at which point the apoptotic cascade is irreversibly activated (Karbowski, Norris, Cleland, Jeong, & Youle, 2006; Wei et al., 2001). Once released, cytochrome c binds to Apaf-1 (apoptotic protease activating factor 1) and caspase-9 to form the apoptosome complex, which then activates the caspase-dependent apoptotic cascade (Kuida et al., 1998; Riedl & Salvesen, 2007; Yoshida et al., 1998). Antagonising the pro-apoptotic function, several Bcl-2 family members as Bcl-2, Bcl-x, Bcl-w, Mcl-1, A1 and Bcl-xL inhibit cell death by preventing the permeabilisation of the mitochondrial membrane. These family members also represent an important survival factor for cancer progression and resistance to anticancer therapies especially when overexpressed (Cory & Adams, 2002). Bcl-2 and Bcl-xL serve a principal role of binding and sequestering specific anti-survival Bcl-2 family proteins (BH3-only molecules) that prevents Bax and Bak activation, while Bcl-xL and Mcl-1 seem to be the key direct antagonists of Bak (Willis et al., 2005).

Cystein proteinases, known as caspases, are also required for apoptosis-related events, being considered essential regulators of cell death and inflammation. Mammalian caspases-2, -3, -6, -7, -8, -9 and -10 represent apoptotic caspases based on their function and they are classified into initiator and executioner members. External death receptors and cell intrinsic signals can both stimulate the apoptosis machinery by irreversibly activating upstream caspases (-2, -8, -9, -10) and consequently the effector members (-3, -6, -7) (Elmore, 2007; Slee, Adrain, & Martin, 1999). Recently, non-apoptotic roles have also been attributed to apoptosis-associated caspases that determine cell differentiation, embryonic

development, cell signalling, motility and compensatory proliferation (Connolly, Jäger, & Fearnhead, 2014; Kuranaga, 2012; Leist & Jäätelä, 2001; Shalini, Dorstyn, Dawar, & Kumar, 2014).

It has been shown that certain apoptotic regulators are critical for normal embryogenesis as seen in digit and mammary gland formation (Meier, Finch, & Evan, 2000). Given the recent research showing additional non-apoptotic functions for cell death signalling during development, this study has focused on analysing the expression pattern of several apoptosis-related proteins in human SG formation, with the aim of understanding what pathways might be involved during gland development.

Material and Methods

Tissue preparation: Major and minor salivary glands from 70 post-mortem human foetuses from natural miscarriages at 4-24 prenatal weeks were obtained from the Medical School of the University of São Paulo under approval of the local Ethical Committee and of the Dental School of the same institution. Foetal SGs were then histologically processed, paraffin-embedded, serial-sectioned and stained with haematoxylin and eosin to study their morphology. Selected specimens (N=15 each; major glands = parotid and submandibular; minor glands = palate and mouth floor) were then utilised for immunohistochemistry, to investigate the expression of Bcl-2, Bax, Bak, Bcl-x, Bcl-xL, Bad, Bid, caspases -6, -7 and -9, cleaved caspase-3, survivin, cytochrome c and Apaf-1, and each protein expression was analysed according to the developmental stages.

Immunohistochemistry: Developing salivary gland samples were sectioned at 4µm and serial sections were deparaffinised, re-hydrated and then submitted to antigen retrieval, as described in Table 1. Each antibody was investigated on all 15 samples, and the reactions were performed in triplicates to verify the reproducibility of the results. The sections were then incubated in 3% aqueous hydrogen peroxide for 15min to quench endogenous peroxidase activity, followed by Protein Block Serum-Free incubation (DakoCytomation, Carpinteria, CA, USA) for 20 min at room temperature to suppress non-specific binding of subsequent reagents. Primary antibodies were added for 2 hours at room temperature (clones, antibody sources and titles in Table 1) and the antigen-antibody complexes were visualized using Advance system (DakoCytomation, Carpinteria, CA, USA) followed by incubation with 3'3 diaminobenzidine tetrachloride (DAB) (DakoCytomation, Carpinteria, CA, USA) for 5min. Sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted with glass coverslip and xylene based mountant. All immunohistochemical assays and analysis were carried out in duplicate. Negative controls were performed by incubation of the specimens with non-immune serum while positive controls were employed according to the manufacture recommendation. Blind qualitative evaluation of all protein expressions was

performed by two experts in SG development using a conventional optical microscope (Olympus E330).

Results

In order to assess the activity of the intrinsic apoptotic pathway during human SG development, the expression of key proteins involved in this process were investigated. This study included pro- and anti-apoptotic members of the Bcl-2 family, effectors and initiator caspases, apoptosome complex components and the apoptotic inhibitor survivin. All results were scored in a semi-qualitative analysis summarised in Tables 2, 3 and 4, which describe the subcellular localisation of each protein according to the developmental stage of the human SGs. It is worth noting that human SG formation is very heterogeneous and consists of mixed stages from early gestational age. Therefore this study focused on characterising and scoring each protein expression pattern according to developmental stages rather than type of gland or gestational age. Representative images of selective groups of proteins are shown in Figures 2 to 5.

Morphological comparison between mouse and human SG developmental stages can be visualised in Figure 1, showing the similarity of the gland initiation and branching morphogenesis. During development formation of the first epithelial bud results from an invagination of the oral epithelium (OE) towards the underlying mesenchyme (Fig 1A, 1F, 1G, arrows). As proliferation continues, these epithelial buds progressively elongate and start forming long stalks at the pseudoglandular stage, where the gland starts to branch and the lumen space starts to form (Fig 1B, 1H, 1I, arrows). This is followed by the cavitation stage, where the glands are more branched and the ducts well luminised (Fig 1C, 1J, 1K, arrows). Finally the glands undergo differentiation, displaying acinar secretory units (Fig 1D, 1L, 1M, 1E, 1N, arrowheads) and the presence of multi-layered excretory ducts (Fig 1O) at the terminal bud stage.

We first investigated the expression of the pro-apoptotic proteins Bak, Bax, Bad and Bid, and anti-apoptotic regulators Bcl-2, Bcl-x and Bcl-xL represented the Bcl-2 family members. Semi-qualitative results according to each developmental stage are summarised in Table 2 and the expression patterns of all positive Bcl-2 family members during human SG development are illustrated in Figure 2. Bcl-2 was completely negative in all specimens, while Bad and Bid showed positivity in one isolated case (N = 1/15). Pro-apoptotic proteins Bax and Bak and apoptotic antagonists Bcl-x and Bcl-xL were observed during all developmental stages (Fig 2). Although Bax was mainly cytoplasmic in all ductal structures (Fig 2A to 2E), few central cells had Bax localised to their nuclear compartment within the presumptive lumen area of the ducts (Fig 2B, 2C, arrows). Detail of both subcellular localisations is illustrated in Fig 2E, where some central ductal cells showed nuclear expression (arrows). Bak in contrast revealed strong nuclear positivity within the presumptive ducts (Fig 2F, 2G, 2H, arrows), and cytoplasmic expression was exclusive at the terminal bud stage (Fig 2I). Both pro-apoptotic markers exhibited strong expression in the cytoplasm of collecting duct cells (Fig 5A, 5B, arrowheads), although Bax was also observed in the nucleus (Fig 5B, arrow).

Antagonistic regulators of intrinsic apoptosis Bcl-x and Bcl-xL were also observed during human SG development, however these two proteins showed opposite expression patterns. Bcl-x had its expression mainly concentrated within the cytoplasmic regions of ductal cells at all developmental stages (Fig 2K to 2O) with occasional nuclear positivity neighbouring the luminal area (Fig 2L, 2M, arrows). At the terminal bud stage the cytoplasmic expression of Bcl-x was more evident in the luminal epithelial layer of more developed ducts (Fig 2N, arrowhead) while small ducts near the acini exhibited discrete nuclear staining (Fig 2N, 2O, arrow). In contrast, Bcl-xL demonstrated more evident nuclear expression within the presumptive luminal areas (Fig 2N, 2O, arrows) with weak cytoplasmic positivity at the initial stage (Fig 2P) and complete absence within intercalated and striated

ducts at terminal bud stage (Fig 2S). In collecting ducts, however, it was strongly expressed mainly in the nuclei (Fig 5D).

Caspases-3, -6 and -7 were included in this study as representing the most important effector caspases of the apoptotic pathway, while caspase-9 denoted the initiator protease. Semi-qualitative analysis of the results obtained was correlated according to SG developmental stages in Table 3. Caspase-3 protein expression was assessed in its activated form as a standard hallmark for detecting active apoptosis. Interestingly, cleaved caspase-3 was completely negative in our analysis (Fig 3A to 3D). Initiator caspase-9 and effector caspases-6 and -7 in contrast were detected during almost all stages (Fig 3).

Analysis of caspases-6 expression showed nuclear localisation from bud to canalicular stage within the presumptive lumen areas (Fig 3E to 3I). At the terminal bud stage, cytoplasmic expression became more prominent within intercalated ducts (Fig 3H, arrowhead), while caspase-6 remained mainly nuclear in striated and collecting ducts (Fig 3H, arrow, Fig 5E). Effector caspase-7 showed more pronounced nuclear than cytoplasmic expression (Fig 3J to 3N), particularly at the pseudoglandular stage (Fig 3K, arrow). More developed ducts also showed caspase-7 mainly within nuclear compartments (Fig 5F, arrow). Characteristic apoptotic nuclei were detected in association with nuclear caspase-7 in few samples (Fig 3N, arrow). Similar to caspase-6, initiator caspase-9 was present only in the nuclei of ductal cells at initial stages (Fig 3O, 3P). Subtle and localised cytoplasmic expression was observed at the canalicular stage (Fig 3Q) and within intercalated ductal cells at the terminal bud stage (Fig 3R, arrowhead). The prevalent nuclear expression of caspase-9 remained stronger in collecting ducts (Fig 5G).

As part of the apoptosome complex, the expression of cytochrome c and Apaf-1 through SG development were analysed and summarised in Table 4. Apaf-1 expression was more prevalent in the nuclei of ductal cells at all developmental stages (Fig 4A to 4D), being only observed in the cytoplasm within intercalated ductal cells at terminal bud stage (Fig 4D,

arrowhead). Collecting ducts also showed more nuclear Apaf-1 (Fig 5H, arrow). In contrast, cytochrome c was exclusively cytoplasmic (Fig 4F to 4I), becoming stronger from the pseudoglandular stage. Same expression pattern could be observed in developed collecting ducts (Fig 5I).

The apoptotic inhibitor survivin showed well-defined subcellular expression patterns depending on the developmental gland stage, also described in Table 4. Early developing epithelial buds and stalks showed the predominance of cytoplasmic survivin (Fig 4K, 4L, arrowheads), in contrast to the later stages where nuclear expression is more evident (Fig 4M, 4N, arrows). As more developed structures, collecting ducts also showed a nuclear-dominant positivity for survivin (Fig 5J).

Discussion

The correct regulation of programmed cell death is critical for developmental homeostasis and normal morphogenesis of embryonic tissues (Flusberg & Sorger, 2015). Apoptosis can be triggered by intrinsic and extrinsic factors, the intrinsic pathway involving mitochondrial events driven by Bcl-2 family proteins (Ulukaya, Acilan, & Yilmaz, 2011). In mouse SG formation, apoptosis has been proposed to be important for the removal of central ductal cells in order to originate the lumen space in a process called cavitation (Andrew & Ewald, 2010; Jaskoll, Chen, Min Zhou, Wu, & Melnick, 2001; Melnick & Jaskoll, 2000). This is strongly supported by recent *in vitro* studies where inhibition of caspases prevented duct narrowing and resulted in impaired branching morphogenesis (Teshima et al., 2016).

The developmental pattern of human SGs has already been described as following a similar process compared to the mouse (Lourenço, Uyekita, Lima, & Soares, 2008; Teshima et al., 2011), although little is known about the role of apoptosis during human glandular morphogenesis. The expression of apoptosis-related proteins analysed in this study support a role for programmed cell death in gland development, particularly during lumen formation agreeing with recent research from the mouse (Teshima et al., 2016), however in the human samples apoptosis did not appear to involve the cleavage of the well-known downstream apoptotic regulator caspase-3.

Bcl-2 family proteins are well known as key initiators of the apoptotic cascade (Moldoveanu, Follis, Kriwacki, & Green, 2014), and a balance between anti- and pro-apoptotic members determines cell fate (Flusberg & Sorger, 2015), contributing to normal development and organ shape as shown in several *in vivo* models. Complete absence of both pro-apoptotic proteins Bax and Bak results in either prenatal lethality or severe defects in several tissues after birth (Lindsten et al., 2000). In the absence of Bax, Bak appears to compensate for the loss of Bax, perhaps explaining the lack of lumen phenotype published in Bax mutants (Nedvetsky et al., 2014). Due to compensation it is very difficult to make claims

about the role of apoptosis from the phenotype of single knockouts. Lack of anti-apoptotic factors results in immunodeficiency and renal developmental problems in Bcl-2^{-/-} mice, whereas extensive apoptosis is observed within the liver and hematopoietic systems of Bcl-xL deficient mice (Hakem et al., 1998; Motoyama et al., 1995). Our results particularly showed varied nuclear and cytoplasmic expression of Bax, Bak, Bcl-x and Bcl-xL proteins in human developing SGs, specifically in ductal cells. Although the majority of apoptotic events occur in the cytosol, the nuclear localisation of several apoptotic markers has already been reported in mouse embryo cells and tumor cells, mostly associated with active apoptosis. Studies with immunohistochemistry and electron microscopy revealed prominent nuclear Bax expression in human colon adenocarcinoma where its expression increased after apoptotic stimulation over a short time frame (Gajkowska, Motyl, Olszewska-Badarczuk, & Godlewski, 2001). Similar findings were observed in other tumour cells (Wang, Ding, Chew-Cheng, Yun, & Chew, 1999) in which Bax and Bcl-2 proteins were detected in association with the nuclear matrix. Bax translocation to the nuclear envelope and through nucleus fibrous nucleoplasm was also shown in mouse mammary cells, increasing nuclear and mitochondrial Bax levels after TGF-beta1 apoptotic stimulation (Motyl et al., 2000). The nuclear localisation of these proteins in correlation with their apoptotic activity supports our findings, where prominent nuclear expressions of Bax, Bak and Bcl-xL were particularly found in presumptive duct areas, agreeing with the hypothesis that apoptosis regulates SG luminal opening. The pro-apoptotic activity in lumen formation seems to be mainly associated with Bak control due to its more pronounced nuclear localisation compared to Bax, whereas Bcl-xL modulates apoptosis during initial lumen opening and Bcl-x takes place only at the latest stage.

The Bcl-2 homologue pro-apoptotic proteins, Bad and Bid, induce apoptosis by extrinsic activation from the cytoplasmic membrane. They transmit cell death signalling through caspase-8 (H. Li et al., 1998; Luo, Budihardjo, Zou, Slaughter, & Wang, 1998). Their absence in our specimens strongly suggests that the apoptotic activation in human gland development depends only on cell intrinsic signalling, confirmed by prominent Bax and Bak expressions

during all stages. Bid-deficient mice are also resistant to Fas-induced hepatocellular apoptosis, although intrinsic stimulation was not shown to result in abnormality, supporting the requirement for Bid in apoptosis only through extrinsic stimuli (Yin et al., 1999). Similarly, double Bax and Bak deficient mice illustrated resistance to both external (such as Fas-induced apoptosis and tBID-induced apoptosis) and intrinsic stimuli (such as growth factor deprivation and ultraviolet radiation), evidencing their requirement to activate both apoptotic pathways (Wei et al., 2001). Bad and Bid although negative in the majority of samples did show positivity in one foetal SG. Our samples were obtained from natural miscarriages and it is possible that this sample had activated the extrinsic pathway for an unknown reason, such as deprivation of nutrition.

Caspases are considered essential apoptotic regulators of both apoptotic pathways and they are found in latent form in different subcellular compartments, being activated by distinct mechanisms such as proteolysis, oligomerisation or binding to membrane receptors or regulating factors (Hyman & Yuan, 2012; Ramuz et al., 2003). Specific subcellular localisation of caspases implies distinct roles during development according to the cell type. Nuclear expression of executioner caspase-7 has been reported to indicate active apoptotic functions during osteogenesis of tooth and jaw development and within the primary enamel knot (Leist & Jäättelä, 2001; Matalova, Svandova, & Tucker, 2012; Matalova, Vanden Berghe, et al., 2012; Miura, 2012; Svandova et al., 2014). Similarly, our results demonstrated prevalent nuclear caspase-7 expression during ductal formation, especially near presumptive luminal areas. Moreover caspase-7 was observed within the characteristic apoptotic condensed nuclei (picnotic nuclei) in the central lumen area, suggesting apoptotic activity in these regions. As an executioner member in the caspase machinery, it is considered that caspase-7 shares properties with caspase-3 as shown in *in vivo* models where development was only impaired in double knockout mice, while the presence of caspase-7 compensates the lack of caspase-3 in single knockout (Lakhani et al., 2006). In addition, analysis of caspase-3 deficient mice illustrates that components of the death pathway act in a tissue/cell type-

specific and stimulus-specific manner (Woo et al., 1998). Detection of cleaved caspase-3 is currently the apoptotic activation hallmark, although alternative caspase-independent cell death pathways have been reported (Leist & Jäättelä, 2001). Our data suggests that caspase-3 might not be an accurate marker to identify apoptotic activity on its own. Negative expression of cleaved caspase-3 revealed in our study indicates therefore that caspase-7 is the main effector caspase in human SG development in contrast to previous reports in mouse models (Jaskoll et al., 2001).

Unlike caspase-3 and -7, caspase-9 is an upstream regulator that comprises part of the apoptosome complex with Apaf-1 and cytochrome c proteins. Caspase-9 therefore represents an important intrinsic apoptotic receptor (Riedl & Salvesen, 2007). Previous findings have shown the requirement of caspase and Apaf-1 in embryogenesis, illustrating that their deficiency promotes defects in mouse olfactory and neural development correlated with apoptotic and non-apoptotic functions (Ohsawa et al., 2010). The absence of initiator caspase-9 displayed analogous but more severe *in vivo* effects than executioner caspase-3 in brain development (Hakem et al., 1998; Kuida et al., 1998). Additionally, mice lacking cytochrome c have also shown developmental defects in apoptosis, being more sensitive to intrinsic and extrinsic cell death signals (Li et al., 2000).

Subcellular localisation of caspase-9 was previously described in mammary epithelial cells, which suggested the epithelium specificity of this protein and associated its nuclear expression with intrinsic apoptotic activation (Ritter et al., 2000). Other studies with breast cell cultures also reported caspase-9 translocation upon cell stress-induced apoptosis in order to disassembly the nuclear-cytoplasmic barrier that allows caspase-3 function. Importantly, activation of caspase-9 was required for caspase-3 to enter the nucleus, whereas the nuclear translocation of caspase-9 did not require activation of caspase-3 (Faleiro & Lazebnik, 2000). Our data comparing nuclear and cytoplasmic expression agrees with these findings from mammary glands, particularly the finding that caspase-9 can be translocated to the nucleus in the absence of any caspase-3.

Given that Apaf-1 is part of the apoptosome complex with caspase-9, Apaf-1 has been shown to be redistributed to the nucleus during intrinsic-induced apoptosis in fibroblasts (Ruiz-Vela, González de Buitrago, & Martínez-A, 2002). In contrast a diffuse cytochrome c was observed in the cytoplasm (Ruiz-Vela et al., 2002). A similar nuclear and cellular localisation of the apoptosome complex members was identified in our study, which may represent an early apoptotic hallmark in human SG development.

Additional roles for caspases have recently been suggested, regulating compensatory proliferation of neighbouring cells, cell-fate determination, stem cell maintenance, tissue regeneration, actin-skeleton reorganisation determining cell shape, immune system and promotion of different types of programmed cell death (Abraham & Shaham, 2004; Kuranaga, 2011). Prominent cytoplasmic expression of caspases-6, -7 and -9 in human glands indicates the existence of potential non-apoptotic roles of these caspases at certain developmental stages. Expression of the effectors caspase-6 and -7 indicated complementary functions during human SG development, as caspase-6 expression was mainly concentrated within more developed ducts at later stage terminal bud, while caspase-7 was more evident at earlier stages.

Apoptosis can also be controlled by several endogenous caspase inhibitors part of the inhibitors of apoptosis proteins (IAP) family. Survivin is the smallest member of the IAP family and is also known as baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5). It is abundantly expressed in foetal development and tumour cells in humans, while rarely present in differentiated tissues. The main role of survivin is to control apoptosis by inhibiting Bax and the extrinsic Fas pathways and binding to caspase-3 and -7, although additional roles in cell division have been described (Chi et al., 2014; Salvesen & Walsh, 2014). Survivin expression has been reported during mouse embryonic submandibular gland development flanking the lumen forming regions (Jaskoll et al., 2001). Given that survivin has both pro and anti-survival functions, they suggested that nuclear survivin played a role in anti-apoptotic activity, where it translocates to form the Cdk4/survivin complex. Survivin expression was

quantitative and qualitatively higher at later stages, indicating it is a key survival factor of epithelial cells surrounding forming ducts and terminal bud lumina (Jaskoll et al., 2001). Our results in human glands similarly showed prevalent nuclear localisation of survivin at later stages, where apoptosis in the ducts seems to be more controlled once the lumen space has already formed.

This work has evidenced the protein expression profile of several apoptosis-related regulators during human salivary gland morphogenesis, supporting that apoptosis is important for SG lumen formation (Melnick & Jaskoll, 2000) however these molecules are also likely to have other non-apoptotic functions within the gland (Fan & Bergmann, 2008). Emerging research in developmental biology is revealing new insights into salivary gland formation, especially with the advantage of more transgenic lines available, indicating indeed new perspectives of apoptotic roles in development. Distinct localisation of several apoptotic regulators may extend their potential sites of action and collaborate in additional roles essential for normal development but also for tumour progression. Hence revealing specific biological processes in SG morphogenesis may help to define novel targets and activation pathways, uncovering markers and treatments for salivary gland dysfunction and disease.

Acknowledgements

T.H.N. Teshima and S.V. Lourenço were funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - grant number 2011/18865-2 and 2015/02824-6). Collaboration with the UK was funded by a RCUK research partnership award funded by the MRC (MR/M026426/1). The authors declare no potential conflict of interest with respect to the authorship and/or publication of this article. We thank Dr Regina Schultz for kindly assisting in obtaining samples.

T.H.N. Teshima wrote the manuscript, collaborated with experimental processes, extracted and analysed all data. R.C.F. Ianez helped with experimental procedures. C.M. Coutinho-Camillo contributed with study design and proof of reading the article. A.S. Tucker provided writing assistance, analysis of data and proof of reading the article. S.V. Lourenco contributed with study design, data extraction and analysis, writing assistance and proof of reading the article.

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Tables

Primary serum	Clones	Title	Source	Antigen Retrieval
Bak	Polyclonal	1:300	Dako	Citrate buffer, pH 6.0
Bax	Polyclonal	1:400	Dako	Citrate buffer, pH 6.0
Bad (N-term)	Y208	1:500	Epitomics	EDTA, pH 9.0
Bid	BH3	1:100	Epitomics	Citrate buffer, pH 6.0
Bcl-2	124	1:200	Dako	Citrate buffer, pH 6.0
Bcl-xL	7D9	1:2000	Chemicon	Citrate buffer, pH 6.0
Bcl-x	Polyclonal	1:500	Dako	Citrate buffer, pH 6.0
Cleaved caspase-3	Polyclonal	1:600	Cell Signaling	Citrate buffer, pH 6.0
Caspase-6 (C-term)	E180	1:150	Epitomics	EDTA, pH 9.0
Caspase-7	7CSP01	1:150	Chemicon	Citrate buffer, pH 6.0
Caspase-9p10	H83	1:500	Santa Cruz	Citrate buffer, pH 6.0
Apaf-1	Polyclonal	1:30	Novocastra	Citrate buffer, pH 6.0
Cytochrome C	CTC05	1:3000	Chemicon	Citrate buffer, pH 6.0
Survivin	Polyclonal	1:400	Neomarkers	EDTA, pH 9.0

Table 1. Summary of respective primary antibody sources, clones, titles and antigen retrieval method included in this study.

	Pro-apoptotic						Anti-apoptotic					
	Bax		Bak		Bid		Bcl-x		Bcl-xL		Bcl-2	
	C	N	C	N	C	N	C	N	C	N	C	N
Initial Bud	+	++	++	+	0	0	++	+	+	+	0	0
Pseudoglandular	++	+	++	+	0	0	++	+	0	+	0	0
Canalicular	++	+	++	+	0	0	++	+	0	++	0	0
Terminal Bud												
Collecting ducts	++	+	+	0	0	0	++	+	+	++	0	0
Small ducts	+	0	+	+	0	0	+	+	0	0	0	0

Table 2. Semi-qualitative analysis of anti and pro-apoptotic Bcl-2 family proteins expression pattern in human salivary gland development, according to each morphogenetic stage. Cytoplasmic (C) and nuclear (N) expressions are shown separately. ++ = strong expression; + = weak expression; 0 = no expression. Small ducts = striated and intercalated ducts.

	Initiator		Executioners					
	Caspase-9		Caspase-6		Caspase-7		Cl. Caspase-3	
	C	N	C	N	C	N	C	N
Initial Bud	0	+	0	+	+	+	0	0
Pseudoglandular	0	+	0	+	+	++	0	0
Canalicular	+	++	0	+	+	+	0	0
Terminal Bud								
Collecting ducts	+	++	+	++	0	+	0	0
Small ducts	+	+	+	+	+	0	0	0

Table 3. Semi-qualitative analysis of caspase expression pattern in human salivary gland development. Cytoplasmic (C) and nuclear (N) expressions are shown separately. ++ = strong expression; + = weak expression; 0 = no expression. Small ducts = striated and intercalated ducts.

	IAP		Apoptosome			
	Survivin		Apaf-1		Cytochrome C	
	C	N	C	N	C	N
Initial Bud	++	+	0	++	+	0
Pseudoglandular	++	+	+	++	++	0
Canalicular	+	++	+	+	++	0
Terminal Bud						
Collecting ducts	0	++	++	++	+	0
Small ducts	0	++	+	+	0	0

Table 4. Semi-qualitative analysis of Survivin, Apaf-1 and Cytochrome C expression patterns during human SG development. Cytoplasmic (C) and nuclear (N) expressions are shown separately. ++ = strong expression; + = weak expression; 0 = no expression. Small ducts = striated and intercalated ducts.

Figure legends:**Figure 1: Comparative images between mouse and human developmental stages of SG morphogenesis**

(A-E) Mouse SG development.

(F-O) Human SG development.

(A, F, G) Bud stage showing initial growth of the oral epithelium (OE) towards the underlying mesenchyme, forming the first epithelial bud (arrows).

(B, H, I) Pseudoglandular stage showing the beginning of lumen formation with in the epithelial stalks (arrows), and the start of branching morphogenesis.

(C, J, K) More branched epithelial structures at the canalicular stage exhibiting multiple ducts with well defined lumal spaces (C, K, arrows).

(D, E, L, M, N, O) Terminal bud stage presents extensive ducts associated with differentiated secretory units (E, N, arrowhead) that show wide and bright cytoplasm with pyramidal morphology (L, M, N, arrowheads). Luminised excretory duct with multiple epithelial layers at the final stage (O). Histological stainings with hemaetoxilin and eosin (A-D, F-O) and Masson's trichrome (E). Magnifications: E = 40X; A, B, C, D = 100X; J, L = 200X; F, G, H, I, K, M, N, O = 400X. Scale bars = 100µm.

Figure 2: Anti-apoptotic Bcl-xL modulates Bax and Bak activity in human SG development

(A, B, C, D) Bax expression; during early development, nuclear localisation is more prominent in presumptive ductal regions (A, B, C, arrows) while cytoplasmic staining is prevalent at late stage (D) (A, B, C = parotid glands; D = mouth floor minor glands).

(F, G, H, I) Bak expression; evidence of apoptotic activity within ductal structures in expected lumen space area (F, G, arrows) and scarce nuclear expression detected at the terminal bud stage (H) (F, H = parotid glands; G = submandibular gland; I = palatal gland).

(K, L, M, N) Bcl-x expression was mainly cytoplasmic in all developmental stages, occasionally present in the nucleus of epithelial cells near lumen regions (J, K, arrows) (K, L, M = submandibular gland; N = mouth floor).

(P, Q, R, S) Bcl-xL expression shows important nuclear positivity in presumptive lumen areas, especially at pseudoglandular and canalicular stages (N, O, arrows), being completely absent at the latest stage (S) (P, R = parotid glands; Q = submandibular gland; S = palatal glands).

(E, J, O, T) High power of nuclear (arrows) and cytoplasmic (arrowheads) expressions of Bax (E, parotid gland), Bak (J, parotid gland), Bcl-x (O, submandibular gland) e Bcl-xL (T, parotid gland) within developing ducts. Arrows represent nuclear positivity, arrowheads indicate cytoplasmic expression. Magnifications: A-D, F-I, K-N, P-S = 400X; E, J, O, T = 1260X (zoom). Scale bars = 30µm.

Figure 3: Caspase expression profile in human gland development

(A, B, C, D) Cleaved Caspase-3 was negative in all specimens analysed (A, B = submandibular glands; C = parotid gland; D = mouth floor gland).

(E, F, G, H, I) Caspase-6 expression; only nuclear positivity is observed within epithelial stalks during early stages (E, F, arrows), while late stages canalicular and terminal bud showed balanced expression between nucleus and cytoplasm within all ductal network (G, H, arrows and arrowheads). Nuclear expression at early stages can be particularly observed in central cells of the developing stalks (I, arrow) (E, F, G, I = parotid glands; H = mouth floor gland).

(J, K, L, M, N) Caspase-7 expression; effector caspase-7 illustrated mixed nuclear and cytoplasmic expression during all glandular developmental stages (J-M), where nuclear pattern was more prominent at the pseudoglandular stage (J) while cytoplasmic positivity was stronger within more developed ducts at canalicular stage (L, M, arrowheads). High magnification illustrates nuclear caspase-7 within apoptotic cell of developing duct (N, arrow) (J, K, N = submandibular glands; L, M = mouth floor glands).

(O, P, Q, R, S) Caspase-9 expression; similarly to caspase-6, caspase-9 demonstrated exclusive nuclear positivity at early stages (O, P) and localised cytoplasmic expression at later stages (Q, R, arrowheads). High power of developing duct shows nuclear (S, arrow) and cytoplasmic (S, arrowhead) caspase-9 in developing ductal region at later stage (S, arrow) (O, P, Q = submandibular glands; R = mouth floor gland; S = parotid gland). Arrows represent nuclear positivity, arrowheads indicate cytoplasmic expression. Magnifications: A-H, J-M, O-R = 400X; I, N, S = 1260X (zoom). Scale bars = 30µm.

Figure 4: The apoptosome complex is activated during human SG development and nuclear anti-apoptotic survivin is more prominent at later stages

(A, B, C, D, E) Apaf-1 expression; nuclear detection was more prominent during initial stages (A, B, C, arrow) and at later stages the intercalated ducts showed important cytoplasmic expression while striated ducts remained mainly nuclear (C, D, arrowheads). High power shows mixed nuclear and cytoplasmic expression in developing duct (E, arrow and arrowhead) (A, B, C, E = submandibular glands; D = mouth floor gland).

(F, G, H, I, J) Cytochrome c expression; exclusive cytoplasmic positivity was observed for cytochrome c protein during all developmental stages (F, I, J = mouth floor glands; G, H = parotid glands).

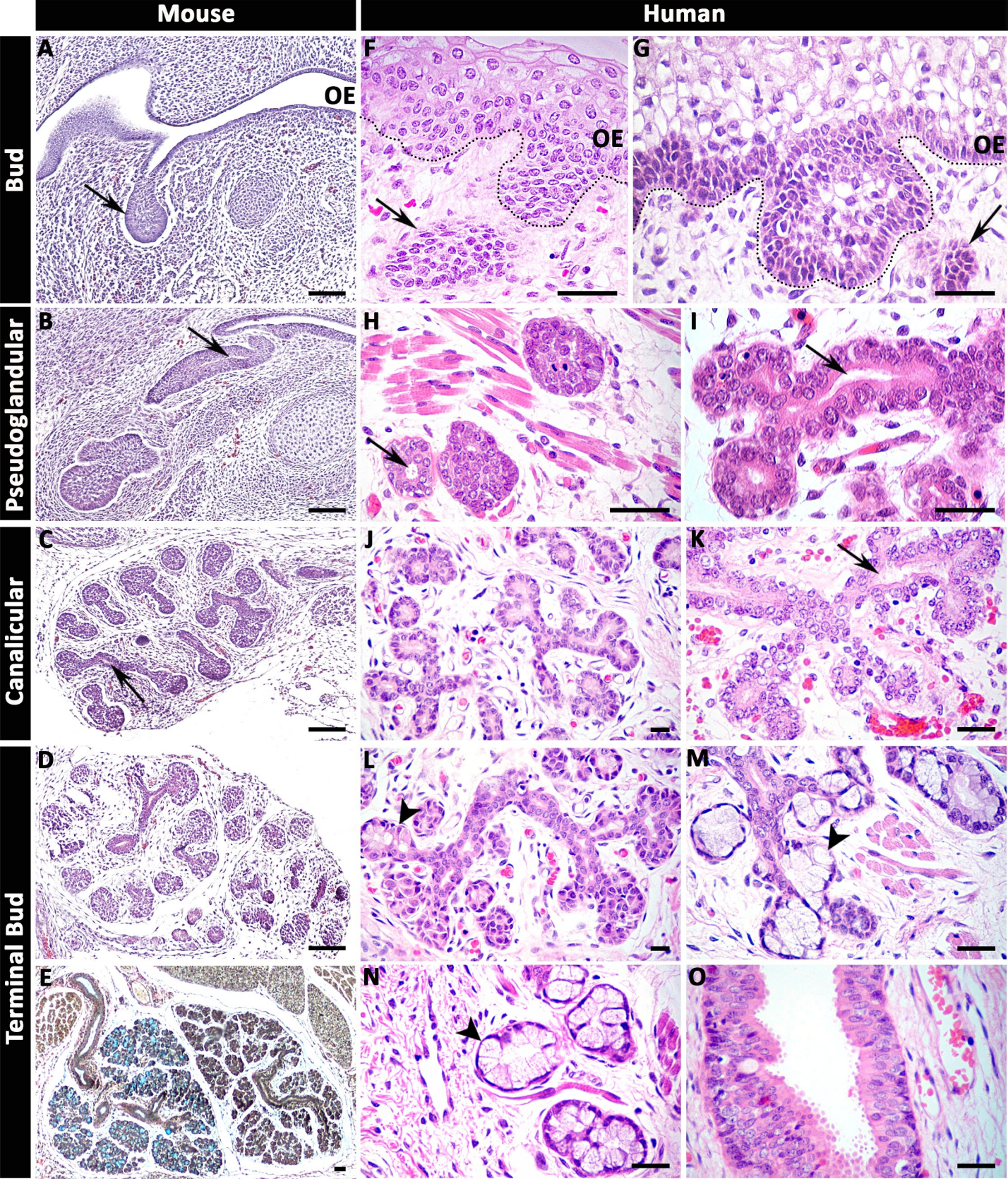
(K, L, M, N, O) Survivin expression; cytoplasmic pattern was more prevalent at initial stages (K, L), becoming nearly absent at later stages (M, N). High power of developing duct shows nuclear survivin in ductal region (O, arrow) and cytoplasmic expression in acinar structure (O, arrowhead) (K, L, M, O = parotid glands; N = mouth floor gland). Arrows represent nuclear positivity, arrowheads indicate cytoplasmic expression. Magnifications: A-D, F-I, K-N = 400X; E, J, O = 1260X (zoom). Scale bars = 30µm.

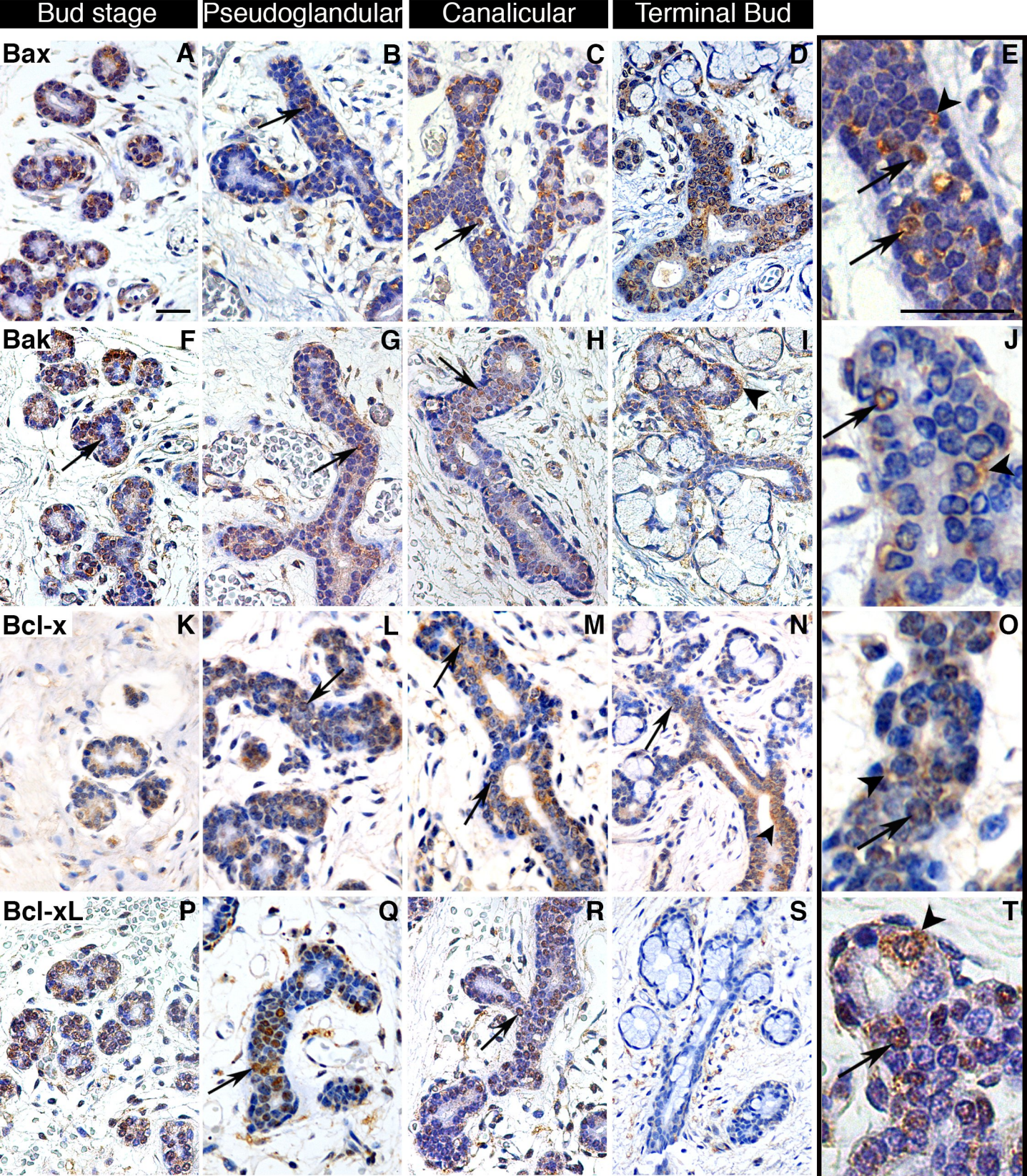
Figure 5: Maintenance of the expression of apoptotic proteins in excretory ducts of human major SG

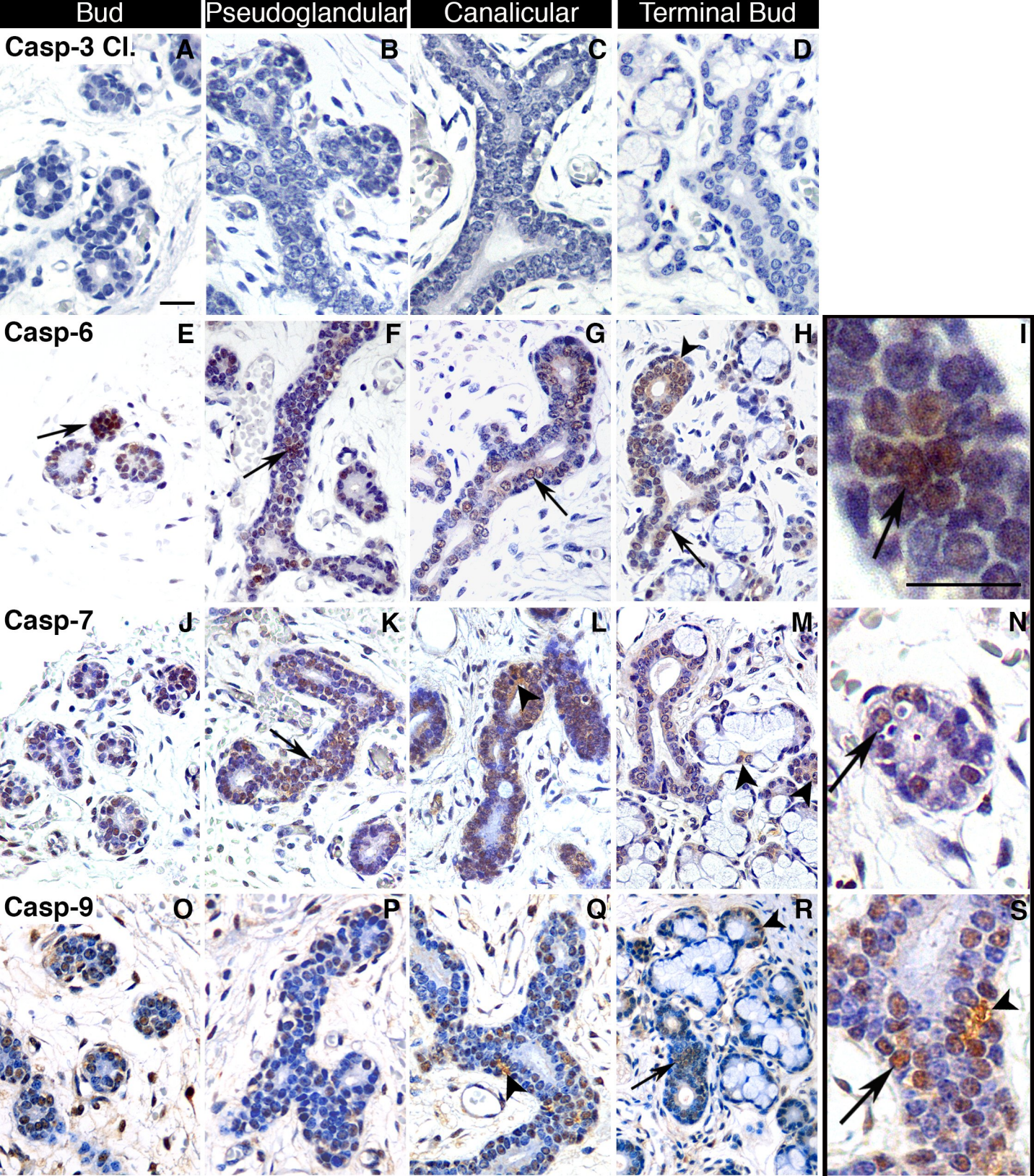
(A, B, C, D) Expression of Bcl-2 family proteins in excretory ducts of submandibular gland at gestational age 20 weeks. Pro-apoptotic Bax was predominantly cytoplasmic (A, arrowhead) with focal nuclear expression (A, arrow), while Bak was only observed in the cytoplasm of basal cells (B, arrowhead). Anti-apoptotic markers Bcl-x and Bcl-xL were both detected in the cytoplasm and nuclei in ductal cells. Bcl-x was however more prominent within the cytoplasm (C, arrowhead) compared to the nuclear region (C, arrow), while Bcl-xL was mostly nuclear found mainly within the luminal layer (D).

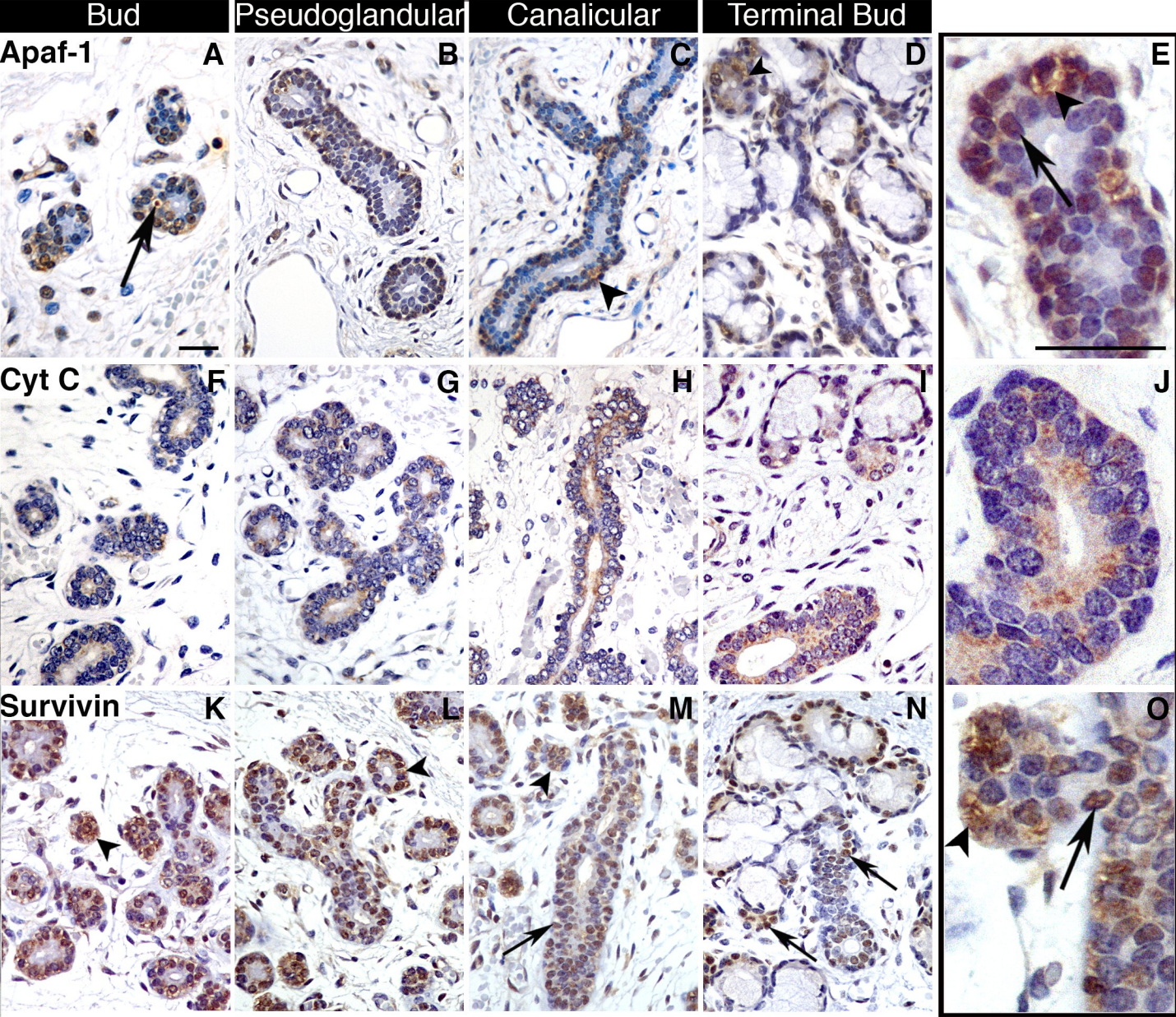
(E, F, G) Expression of caspases-6, -7 and -9 of submandibular gland at gestational age 20 weeks. The expression of all caspases was more prominent in the nuclei of ductal cells, being caspase-7 exclusively found in the nuclei (F, arrow). Caspases-6 and -9 also exhibited weak cytoplasmic expression in few ductal cells (E, G).

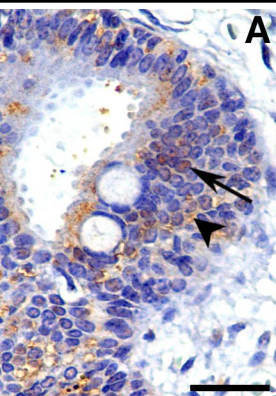
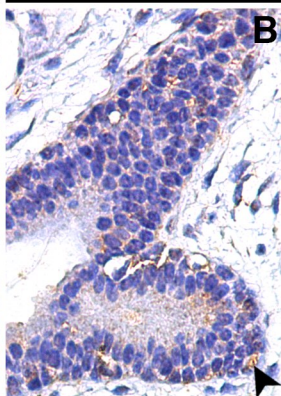
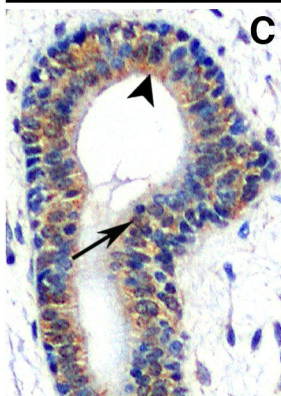
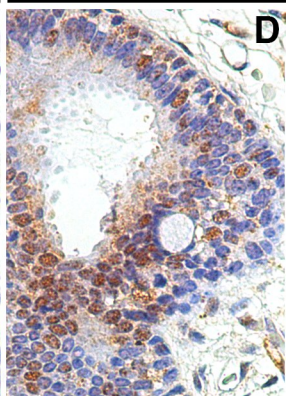
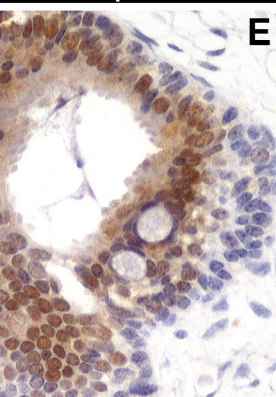
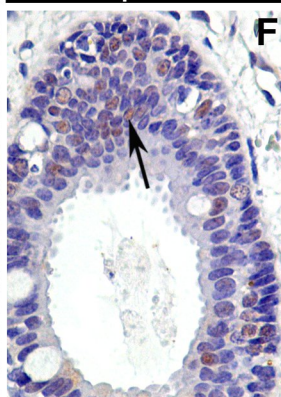
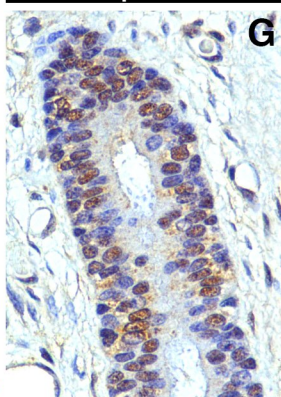
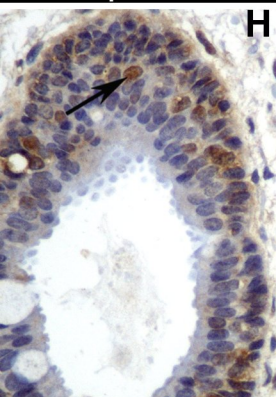
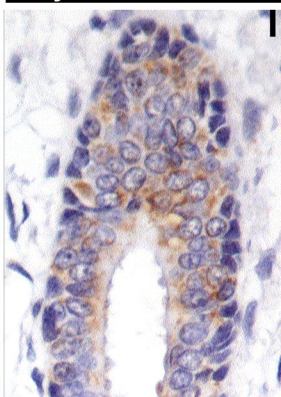
(H, I, J) Mixed expression pattern of Apaf-1, Cytochrome c and survivin in excretory ducts of parotid (I) and submandibular glands (H, J) at gestational age 20 weeks. Apaf-1 was positive in both nuclei and cytoplasm (H), being more nuclear concentrated within luminal cells (H, arrow). Cytochrome c and survivin showed opposite expressions, where Cytochrome c was exclusively cytoplasmic (I) while survivin was only observed in nuclei (J). Arrows represent nuclear positivity and arrowheads indicate cytoplasmic expression. Magnification = 400X. Scale bars = 30µm.









Bax**Bak****Bcl-x****Bcl-xL****Caspase-6****Caspase-7****Caspase-9****Apaf-1****Cytochrome C****Survivin**